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Alpha-lipoic acid supplementation restores the meiotic competency and fertilization capacity of porcine oocytes induced by arsenite

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Arsenite is known as a well-known endocrine disrupting chemicals, and reported to be associated with an increased incidence of negative health effects, including reproductive disorders and dysfunction of the endocrine system. However, it still lacks of the research regarding the beneficial effects of ALA on arsenite exposed oocytes, and the underlying mechanisms have not been determined. Here, we report that supplementation of alpha-lipoic acid (ALA), a strong antioxidant naturally present in all cells of the humans, is able to restore the declined meiotic competency and fertilization capacity of porcine oocytes induced by arsenite. Notably, ALA recovers the defective nuclear and cytoplasmic maturation of porcine oocytes caused by arsenite exposure, including the impaired spindle formation and actin polymerization, the defective mitochondrion integrity and cortical granules distribution. Also, ALA recovers the compromised sperm binding ability to maintain the fertilization potential of arsenite-exposed oocytes. Importantly, ALA suppresses the oxidative stress by reducing the levels of ROS and inhibits the occurrence of DNA damage along with apoptosis. Above all, we provide a new perspective for the application of ALA in effectively preventing the declined oocyte quality induced by environmental EDCs.

KEYWORDS

alpha-lipoic acid, arsenite, meiotic defect, fertilization capacity, oocyte quality, oxidative stress, apoptosis

Introduction

As one of the most well-known Environmental Endocrine Disruptors (EDCs), arsenic is ubiquitous in the environment, arsenic in the drinking water, air, land, and living systems, is considered as one of the top environmental health threats in worldwide (Gao et al., 2021). Arsenic is found in the environment as oxides, with two different oxidation states: arsenite (As (III), as H_3AsO_3) and arsenate (As (V), as $HAsO_4^{2-}$). In the aqueous

environment, the oxyanions consisting of arsenite species and arsenate species predominate. Arsenite species tend to predominate in groundwater under reducing conditions, whereas arsenate species are more frequently found under oxidizing conditions. The mobility of arsenic compounds in soils depends on the pH value, the redox potential, organic matter, clay and sand content, and other elements in the soil. It is noteworthy that arsenite is 100 times more toxic than arsenate because of its carcinogenic, teratogenic and mutagenic nature (Firrincieli et al., 2019). Since its high-affinity interaction with the ligand-binding domain of the estrogen receptors alpha, arsenite affects both the male and female reproductive systems. Humans are exposed to arsenite through a variety of ways, including groundwater consumption, diet and inhalation of airborne particulates matter from contaminated soil (Palma-Lara et al., 2020; Wu et al., 2020). Epidemiological data have revealed that arsenite can easily enter the bloodstream once ingested, and transported to the liver and distributed to body's organs and tissues, eventually accumulated in the body (Bourguignon et al., 2017). Previous studies showed that prolonged exposure to arsenite cause peripheral vascular disease, increasing the incidence of lung, kidney, skin and liver cancers (Tryndyak et al., 2020). Arsenite also cause damage to the reproductive system in animals in different ways and to different degrees. For the male reproductive system, arsenite lowers the weight of testicular tissues and reduces the sperm counts along with the lower sperm motility, chromosomal aberrations and morphological sperm abnormalities (Wu et al., 2020). As for female, arsenite causes female masculinization in rats, ultimately impairing their normal reproductive function (Souza et al., 2020). In addition, arsenite delays the initiation of puberty in rats and increases the incidence of spontaneous abortion and stillbirth, accompanied with the presence of the antioxidant enzyme activity and sex hormone concentrations in the ovaries and uterus (Quansah et al., 2015). Female fertility is regulated by both the female reproductive system and the endocrine system (Yao et al., 2021). Thus, it is susceptible to the exposure of EDCs in the environment (Green et al., 2021). There is now overwhelming evidences that arsenite causes the accumulation of ROS and reactive nitrogen species (RNS) in somatic cell and male germ cells (Wang et al., 2013). ROS play important roles in the reproductive process including ovulation, nevertheless, excessive ROS and RNS attack important macromolecules and organelles inducing mitochondrial dysfunction, and thereby leading to the cell apoptosis (Guidarelli et al., 2017). For example, excess oxygen free radicals can damage oocytes at a genetic level through oxidative stress, causing infertility (Barati et al., 2020). However, the underlying mechanisms concerning how arsenite impacts the meiotic competence and fertilization capacity of porcine oocytes still remain unknown.

Alpha-lipoic acid (ALA), a natural care product in the health market, is a strong antioxidant present in all types of prokaryotic and eukaryotic cells (Di Nicuolo et al., 2021). ALA participates in

aerobic metabolism, converting blood sugar (glucose) into energy consuming the oxygen (Erickson et al., 2020). In particular, ALA exerts the effects by scavenging free radicals, metal ion chelation and antioxidant recycling. Besides, ALA promotes the effectiveness of endogenous antioxidants such as glutathione and enhances their capacity to scavenge free radicals (Solmonson and DeBerardinis, 2018; Salehi et al., 2019). ALA has been identified to effectively scavenge ROS and eliminate the damage of the reproductive systems exposed to heavy metals, thereby ameliorating fertility in rats (Prathima et al., 2018).

In the current paper, porcine oocytes were used as the research model to study whether the application of ALA could prevent the declined oocyte quality induced by arsenite, because there are many similarities between porcine oocytes and human oocytes in aspects of physiological metabolism and pathogenesis (Brazert et al., 2020). And here we provide several lines of evidence demonstrating that ALA ameliorates the quality of oocytes induced by arsenite by suppressing oxidative stress-induced DNA damage and apoptosis, which would compromise the critical regulators involved in oocyte maturation and fertilization.

Materials and methods

Antibodies

Mouse monoclonal anti- γ H₂AX antibody were obtained from Abcam (Cambridge, MA, United States); Mouse monoclonal anti- α -tubulin FITC antibody, peanut agglutinin (PNA) -FITC, anti-actin antibody and anti-acetylation- α -tubulin (Lys-40) antibody were obtained from Sigma (St. Louis, MO, United States); Rabbit polyclonal anti-GAPDH antibody was obtained from Servicebio (Wuhan, China); Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) were obtained from Thermo Fisher Scientific (Waltham, MA, United States); HRP-labeled Goat Anti-Rabbit IgG (H + L) was obtained from Beyotime (Shanghai, China).

Arsenite treatment and ALA supplementation

Arsenite and ALA were obtained from Sigma (St. Louis, MO, United States). The drugs were both dissolved and diluted in dimethyl sulfoxide (DMSO). Arsenite was further diluted into final working concentrations with *in vitro* medium (5 μ M, 10 μ M, 20 μ M and 40 μ M). ALA was further diluted into final working concentrations with *in vitro* medium (5 μ M, 10 μ M, 25 μ M and 50 μ M). The incubation medium should contain no more than 0.1% DMSO because of the cytotoxicity of DMSO itself. These

doses were picked on the basis of data reported by previous examinations (Navarro et al., 2006; Makvandi et al., 2019).

Experimental design

In this experiment, porcine oocytes were used as experimental model to explore the protective role of ALA against arsenite-induced meiosis defects since pigs share many physiological similarities with humans. The oocytes were randomly assigned to three groups as follows: 1) The control group; 2) The arsenite-treated group was supplemented with 40 μ M arsenite; 3) The ALA-supplemented group was supplemented with 25 μ M ALA. The concentration of arsenite (40 μ M) and ALA (25 μ M) was determined based on previous study and the effect of them on the first polar body extrusion. Each group contains at least 50 oocytes. The collected COCs were incubated in a preheated medium with arsenite and/or ALA and cultured in 38.5°C 5% CO₂ incubator. After 30 h culture, oocytes developed to MI stage, and after 42 h of culture, oocytes developed to MII stage.

Experiment 1. The effects of ALA on the developmental competence and fertilization potential of arsenite-exposed porcine oocytes. The rates of the cumulus expansion of the COCs, the first polar body extrusion (PBE), fertilization and blastocyst were evaluated in control, arsenite-exposed and ALA-supplemented groups.

Experiment 2. The effects of ALA on the nuclear maturation of arsenite-exposed oocytes. The spindle morphology, chromosomes alignment and microtubule stability were tested in control, arsenite-exposed and ALA-supplemented groups.

Experiment 3. The effects of ALA on the cytoplasmic maturation of arsenite-exposed oocytes. The actin dynamics, localization of cortical granules and mitochondrial integrity were assessed in control, arsenite-exposed and ALA-supplemented groups.

Experiment 4. The effects of ALA on the sperm binding ability of arsenite-exposed oocytes. The sperm binding to the zona pellucida were tested in control, arsenite-exposed and ALA-supplemented groups.

Experiment 5. The molecular mechanism of ALA preventing the negative effect induced by arsenite. The level of ROS, DNA damage and apoptosis were determined in control, arsenite-exposed and ALA-supplemented groups.

Porcine oocytes collection and *in vitro* maturation

To obtain high-quality oocytes, the ovaries were obtained and selected in slaughterhouses. The ovaries were conserved in 0.9% saline with penicillin (200,000 units) and

streptomycin (250,000 units) at 38.5°C within 2 h. After washed for 3 times with fresh saline, the cumulus-oocyte complexes (COCs) were aspirated using a disposable syringe from the follicles on the outside layer of the ovaries. The COCs with a compact cumulus cells were selected and then moved into the maturation medium for the *in vitro* maturation. The maturation medium was made up on the basis of TCM-199 supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 0.5 μ g/ml PG 600, 100 μ l/ml of PFF, 50 μ l/ml FBS, 10 ng/ml EGF and 0.1 mg/ml L-cysteine (Chen et al., 2021). COCs were moved into a drop of 50 μ l maturation medium covered with mineral oil at 38.5°C and 5% CO₂ for 28–30 h to metaphase I stage and for 42–44 h to metaphase II stage.

Immunofluorescence staining and confocal microscopy

Denuded oocytes (DOs) were obtained by transferring COCs to 200 μ l of 1 mg/ml hyaluronidase solution for 15 min to remove the granulosa cells. DOs were fixed in 4% paraformaldehyde (PFA) at room temperature for 20 min, then transferred into 0.5% Triton X-100 for 30 min for permeabilization. After blocking with blocking buffer (2% BSA-supplemented DPBS) for 1 h at room temperature, DOs were then incubated with primary antibody overnight at 4°C. Then the oocytes were incubated with secondary antibody for 1 h at room temperature after washed 3 times in DPBS. Then oocytes were counterstaining with propidium iodide (PI) or DAPI to stain nuclei for 10 min. Lastly, the samples were observed and imaged under a confocal microscope (Olympus, Japan).

Western blot analysis

A total of 50 porcine oocytes for each group were collected and lysed in RIPA/6 x loading buffer (Beyotime Institute of Biotechnology, China) containing protease inhibitors, which heated at 95°C for 5 min. Proteins were separated on 6% future PAGE protein prep gel (Nanjing ACE Biotechnology Co., Ltd, China) and then transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes containing the target protein were blocked in Tris-buffered saline Tween 20 (TBST) containing 5% skimmed milk powder for 3 h at room temperature to block up nonspecific sites. And then PVDF membranes were incubated with anti-acetylated α -tubulin antibody (1:8000) or anti-GAPDH antibody (1:4000) overnight at 4°C. After washed 3 times in TBST, the PVDF membranes were transferred into secondary antibodies and incubated for 2.5 h at room temperature. Chemiluminescence was performed with ECL Plus (New

Cell & Molecular Biotech Co., Ltd, China) and signals were acquired by Chemiluminescence Imaging System 398 (Uvltec Ltd, Cambridge, United Kingdom).

In vitro fertilization

Fresh semen from 20-month-old Landrace Pigs were diluted and resuspend in IVF medium to a final concentration of 0.25×10^6 sperms/ μl , followed by the oocytes were incubated at 38.5°C for 0.5 h. Then 15 MII oocytes and 50 μl sperms were incubated together in fertilization medium for a total of 5 h at 38.5°C , 5% CO_2 . After washed 3 times to remove the extra sperms, the oocytes were cultured in 4-well dishes with 400 μl of embryo medium at 38.5°C , 5% CO_2 . With the addition of sperm, the 2-cell embryo at 32 h was scored as successful fertilization. The blastocyst was observed and counted at 144 h to view the embryonic development.

Sperm binding assay

Fresh semen were diluted and resuspend in IVF medium to a final concentration of 0.25×10^6 sperms/ μl , followed by the oocytes were incubated at 38.5°C for 0.5 h. Then 15 MII oocytes and 50 μl sperms were incubated together in fertilization medium for a total of 1 h at 38.5°C , 5% CO_2 . Sperm binding to oocytes were observed using capacitated sperm and two-cell embryos as a negative wash control. After the samples were fixed in 4% PFA for 20 min, sperm heads were stained with DAPI quantified the sperm binding capacity. The number of sperms bound to each oocyte was observed and counted by confocal microscopy. The results reflect the mean \pm SEM from at least three independently obtained samples, each containing 10–15 porcine oocytes/embryos.

Determination of mitochondrial distribution

Oocytes were incubated in the maturation medium containing 500 nM cell permeant MitoTracker Red CMXRos (Thermo Fisher, United States) to evaluate the distribution of active mitochondria. Oocytes should be incubated in 38.5°C 5% CO_2 incubator for 30 min. Then the oocytes were washed four times with the maturation medium and observed under the confocal microscope (Olympus, Japan).

Determination of ROS generation

To determine the levels of intracellular ROS production, oocytes were transferred into medium with the fluorescent

probe [dichlorofluorescein (DCFH-DA)], according to the manufacturer's instruction (Beyotime Institute of Biotechnology, China). The DCFH-DA working concentration were 10 μM and the incubation time were 30 min. Oocytes should be incubated in 38.5°C 5% CO_2 incubator for 30 min. After washing three times in DPBS containing 0.3% PVP, the fluorescent intensity of the oocytes were observed under the confocal microscope (Olympus, Japan).

Annexin-V staining

The oocytes apoptosis were detected with the Annexin-V-FITC apoptosis kit (Vazyme, China). Specifically, after washed three times in DPBS, the denuded oocytes were transferred into mixture containing Annexin-V-FITC and the binding buffer (1: 9) for 30 min in the dark for the incubation. Then the oocytes were washed three times in DPBS and observed under the confocal microscope (Olympus, Japan).

Statistical analysis

Data are representative of at least three independent repetitions, and data values are expressed as mean \pm SEM. The number of oocytes observed is labeled in parentheses (n). The data were expressed as mean \pm SEM and analyzed by one-way ANOVA, followed by LSD's post hoc test, which was provided by SPSS16.0 statistical software. One-way ANOVA were respectively used for parametric and nonparametric variables. The level of significance was accepted as $p < 0.05$.

Results

ALA improves the developmental ability and fertilization capacity in arsenite-exposed oocytes

To investigate the potential protective role of ALA in the improvement of meiotic competence of porcine oocytes, we first examined the cumulus expansion of the COCs and the occurrence of first polar body extrusion (PBE) of oocytes, both are essential for meiotic maturation and developmental competence (Yin et al., 2019). For this purpose, different concentrations of arsenite (5, 10, 20 and 40 μM) and ALA (5, 10, 25 and 50 μM) were supplemented into the IVM medium, respectively. As shown in Figure 1A, most of the cumulus cells surrounding control oocytes were fully expanded, whereas those in the arsenite-exposed group were partially expanded or not expanded (Figure 1A). Adversely, supplementation of ALA is able to improve the cumulus expansion, especially the concentration of 25 μM , which is selected for the further

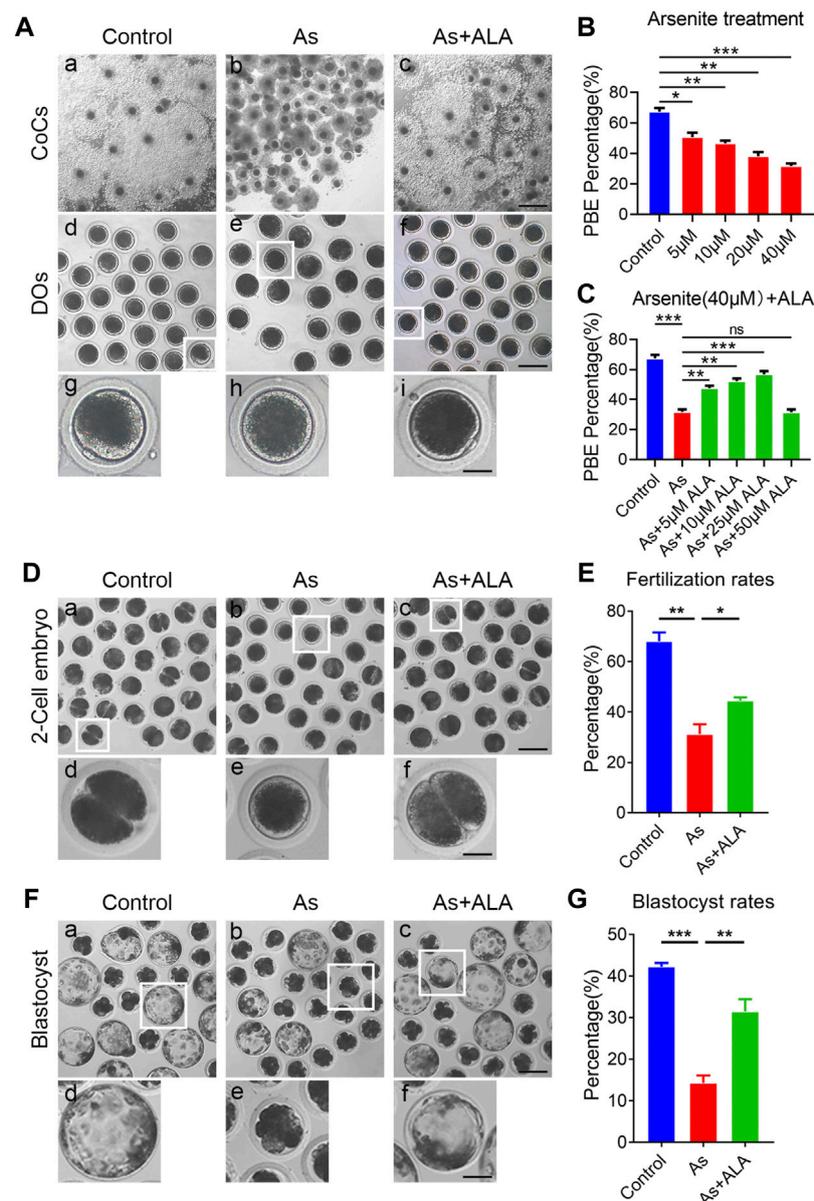


FIGURE 1

Effects of ALA supplementation on the porcine oocyte maturation and fertilization ability in arsenite-exposed oocytes. (A) Representative images of *in vitro* matured oocytes in control, arsenite-exposed and ALA-supplemented groups. Cumulus cell expansion of oocytes and PB1 extrusion were imaged by the inverted light microscope. Scale bar: 350 μm (a-c); 150 μm (d-f); 50 μm (g-i). (B) The rate of polar body extrusion were calculated in control and different concentrations of arsenite-exposed groups (5, 10, 20 and 40 μM) after culture for 44 h *in vitro*. (C) The rate of polar body extrusion were calculated in control, 40 μM arsenite-exposed and different concentrations of ALA-supplemented groups (5, 10, 25 and 50 μM) after *in vitro* maturation. (D) Representative images of 2-cell embryos in control, arsenite-exposed and ALA-supplemented groups. Scale bar: 120 μm (a-c); 40 μm (d-f). (E) The rate of *in vitro* fertilization were calculated in control, arsenite-exposed and ALA-supplemented groups. (F) Representative images of blastocysts developed from the fertilized control, arsenite-exposed and ALA-supplemented groups. Scale bar: 150 μm (a-c); 60 μm (d-f). (G) The rate of blastocysts were calculated in control, arsenite-exposed and ALA-supplemented groups. Data were presented as mean percentage (mean \pm SEM) of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

studies. In addition, the majority of oocytes in the control group undergo normal PBE, but failed to do so following arsenite exposure (Figure 1A). Quantitative analysis showed a dose-dependent decrease in the proportion of PBE after arsenite

treatment compared to the controls (control: $67.4 \pm 2.4\%$, $n = 137$; 5 μM : $50.7 \pm 2.9\%$, $n = 120$, $p < 0.05$; 10 μM : $46.6 \pm 1.8\%$, $n = 127$, $p < 0.01$; 20 μM : $38.1 \pm 2.7\%$, $n = 123$, $p < 0.01$; 40 μM : $31.7 \pm 1.7\%$, $n = 116$, $p < 0.001$; Figure 1B), especially the concentration

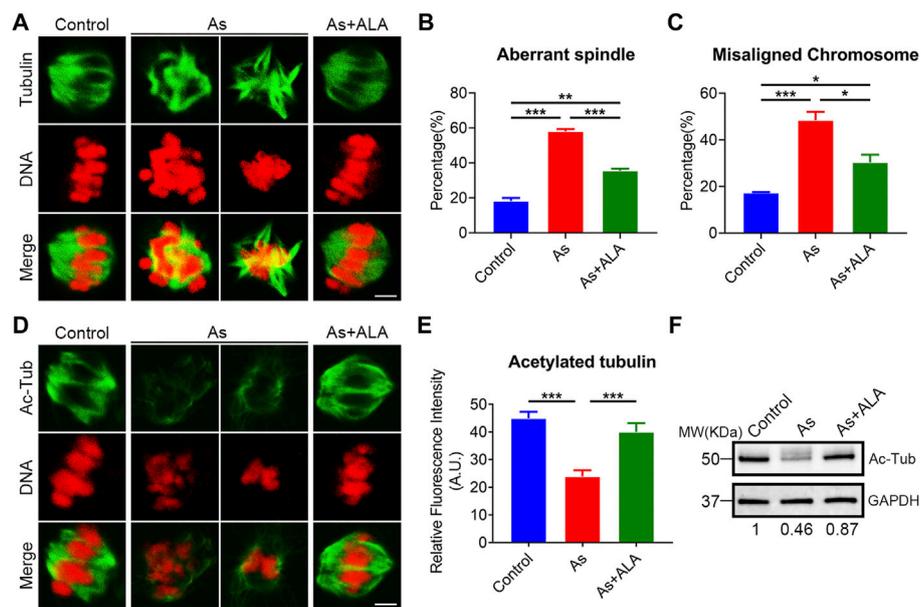


FIGURE 2

Effect of ALA supplementation on spindle assembly, chromosome alignment and microtubule stability in arsenite-exposed oocytes. **(A)** Representative images of spindle morphologies and chromosome alignment in control, arsenite-exposed and ALA-supplemented groups. MI oocytes were immunostained with anti- α -tubulin-FITC antibody and counterstained with propidium iodide (PI). Scale bar, 3 μ m. **(B)** The rate of aberrant spindle were calculated in control, arsenite-exposed and ALA-supplemented groups. **(C)** The rate of misaligned chromosomes were calculated in control, arsenite-exposed and ALA-supplemented groups. **(D)** Representative images of acetylated α -tubulin in control, arsenite-exposed and ALA-supplemented groups. MI oocytes were immunostained with anti-acetyl- α -tubulin antibody and counterstained with propidium iodide (PI). Scale bar, 3 μ m. **(E)** The fluorescence intensity of acetylated α -tubulin was quantified in control, arsenite-exposed and ALA-supplemented groups. **(F)** The acetylation level of α -tubulin were measured by immunoblotting in control, arsenite-exposed and ALA-supplemented groups. The blots were probed with anti-acetyl- α -tubulin and anti-GAPDH antibodies, respectively. Data were presented as mean percentage (mean \pm SEM) of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

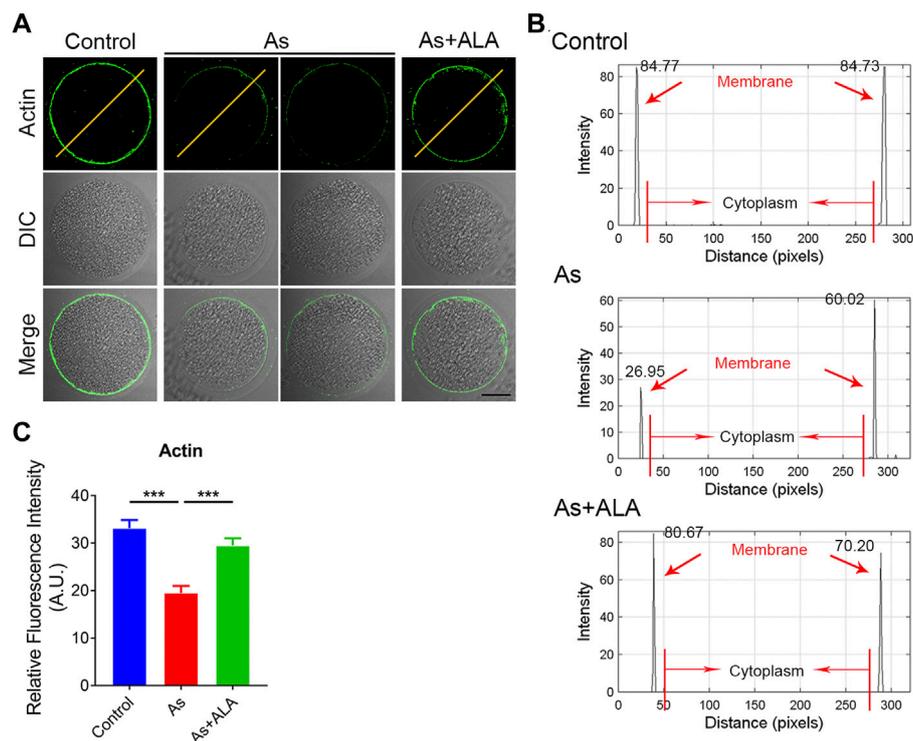
of 40 μ M ($67.4 \pm 2.4\%$, $n = 137$ vs. $31.7 \pm 1.7\%$, $n = 116$, $p < 0.001$; **Figures 1A,C**). Whereas, ALA increased the proportion of PBE of arsenite-exposed oocytes, especially the concentration of 25 μ M ($56.7 \pm 2.2\%$, $n = 201$ vs. $31.7 \pm 1.7\%$, $n = 116$, $p < 0.001$; **Figures 1A,C**). Therefore, we chose the concentration of 40 μ M arsenite and 25 μ M ALA for the follow-up studies because they affected the cumulus expansion of the COCs and the occurrence of PBE most effectively.

Fertilization capacity is also a key indicator reflecting oocytes quality, thus we further evaluated whether ALA could elevate the fertilization potential of oocytes exposed to arsenite (Gadella, 2012). The results showed that the majority of control oocytes were successfully fertilized and developed into two-cell embryos, whereas the fertilization rate of oocytes in the arsenite-exposed group remarkably decreased compared with the controls ($31.2 \pm 3.9\%$, $n = 101$ vs. $68.0 \pm 3.5\%$, $n = 159$, $p < 0.01$; **Figures 1D,E**). As expected, ALA significantly improved fertilization rates of the oocyte exposed to arsenite ($44.4 \pm 1.4\%$, $n = 108$, $p < 0.05$; **Figures 1D,E**). We then further evaluated the subsequent early embryonic development of fertilized oocytes by examining the blastocyst rates. As shown in **Figure 1F**, the blastocyst rates of oocytes in arsenite-exposed group were significantly lower than

that in the control group ($14.3 \pm 1.8\%$, $n = 113$ vs. $42.2 \pm 0.9\%$, $n = 125$, $p < 0.001$; **Figures 1F,G**). As expected, ALA significantly increased the blastocyst rates of the arsenite-exposed oocytes ($31.5 \pm 2.9\%$, $n = 142$, $p < 0.01$; **Figures 1F,G**). The above results indicate that ALA had a beneficial effect on the fertilization ability of oocytes exposed to arsenite and promotes the subsequent embryonic developmental.

ALA restores the spindle assembly, chromosome alignment and microtubule stability in arsenite-exposed oocytes

Studies reported that meiotic arrest is frequently accompanied with the impairment of cytoskeleton structures and functions (Zhou et al., 2019). We thus examined the spindle morphology and chromosomes alignment in oocytes following arsenite and ALA treatment. For this purpose, oocytes were immunostained with anti- α -tubulin-FITC antibodies to visualize the spindle morphology with PI to observe the chromosome alignment. The results showed that, oocytes displayed a typical barrel-like spindle apparatus with a well-

**FIGURE 3**

Effect of ALA supplementation on actin dynamics in arsenite-exposed oocytes. **(A)** Representative images of the localization of microfilaments in control, arsenite-exposed and ALA-supplemented groups. Scale bar, 30 μm . **(B)** The graphs showed the fluorescence intensity profiling of actin filaments control, arsenite-exposed and ALA-supplemented groups. Pixel intensities were measured along the lines which were drawn across the oocytes. **(C)** The fluorescence intensity of actin signals in control, arsenite-exposed and ALA-supplemented groups. Data were presented as mean percentage (mean \pm SEM) of three independent experiments. *** $p < 0.001$.

aligned chromosome on the equatorial plate in the control group. In sharp contrast, various types of aberrant spindle morphologies with misaligned chromosomes were observed in arsenite-exposed oocytes (spindle: $18.2 \pm 1.7\%$, $n = 191$ vs. $58.0 \pm 1.4\%$, $n = 143$, $p < 0.001$; chromosome: $17.3 \pm 0.3\%$, $n = 229$ vs. $48.5 \pm 3.6\%$, $n = 162$, $p < 0.001$; **Figures 2A–C**). As expected, ALA significantly decreased the incidence of abnormal structure of spindle/chromosome (spindle: $35.6 \pm 1.1\%$, $n = 163$, $p < 0.001$; chromosome: $30.3 \pm 3.3\%$, $n = 155$, $p < 0.05$; **Figures 2A–C**).

Normally, structural abnormalities of spindle were frequently associated with the disruption of acetylation level of α -tubulin, an indicator of stable microtubules (Fernandez-Barrera and Alonso, 2018). We then test the acetylation level of α -tubulin porcine oocyte following arsenite and ALA treatment. To this end, oocytes were immunostained with anti-acetylated-tubulin-FITC antibody to observe the acetylation level of α -tubulin with PI to analyze the chromosome alignment. The results showed that the fluorescence intensity of acetylation level of α -tubulin was significantly reduced in the arsenite-exposed oocytes compared to the control group (23.9 ± 2.3 , $n = 23$ vs. 45.0 ± 2.4 , $n = 23$, $p < 0.001$; **Figures 2D,E**), but significantly

raised following ALA supplementation by contrast (40.0 ± 3.2 , $n = 14$ vs. 23.9 ± 2.3 , $n = 23$, $p < 0.001$; **Figures 2D,E**). This observations were further confirmed with western blotting analysis (**Figure 2F**). Above all, these results indicate that ALA supplementation restore the spindle structure impaired by arsenite probably through the maintenance of acetylation level of α -tubulin.

ALA restores the actin dynamics in arsenite-exposed oocytes

Actin assembly is crucial for spindle assembly and cortical polarization during meiosis (Stricker et al., 2010). Thus, we next assessed the actin dynamics following arsenite and ALA treatment. To this end, anti-actin antibody was used to label the F-actin. As shown in **Figure 3A**, actin filaments of oocytes from the control group were concentrated uniformly on the plasma membrane with robust signals. Nevertheless, the accumulation of actin signals on the plasma membrane was diminished or completely lost in arsenite-exposed oocytes.

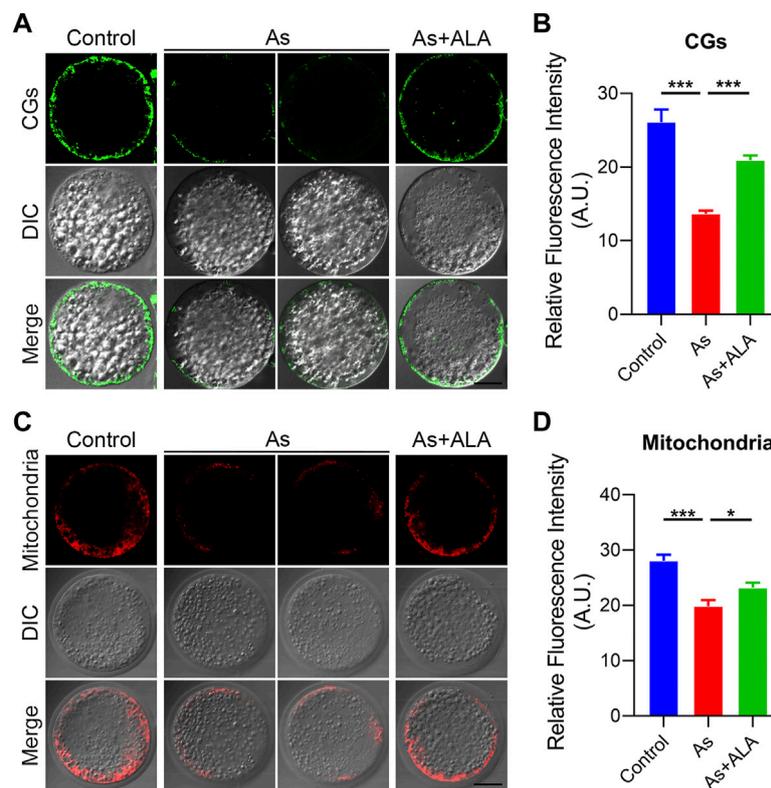


FIGURE 4

Effect of ALA supplementation on the localization of cortical granules and mitochondrial integrity in arsenite-exposed oocytes. (A) Representative images of the localization of cortical granules in control, arsenite-exposed and ALA-supplemented groups. LCA-FITC was used to show the CGs in the oocytes. Scale bar, 30 μ m. (B) The fluorescence intensity of cortical granules in control, arsenite-exposed and ALA-supplemented groups. (C) Representative images of the mitochondrial integrity in control, arsenite-exposed and ALA-supplemented groups. Scale bar, 30 μ m. (D) The fluorescence intensity of mitochondrial signals in control, arsenite-exposed and ALA-supplemented groups. Data were presented as mean percentage (mean \pm SEM) of three independent experiments. * p < 0.05, *** p < 0.001.

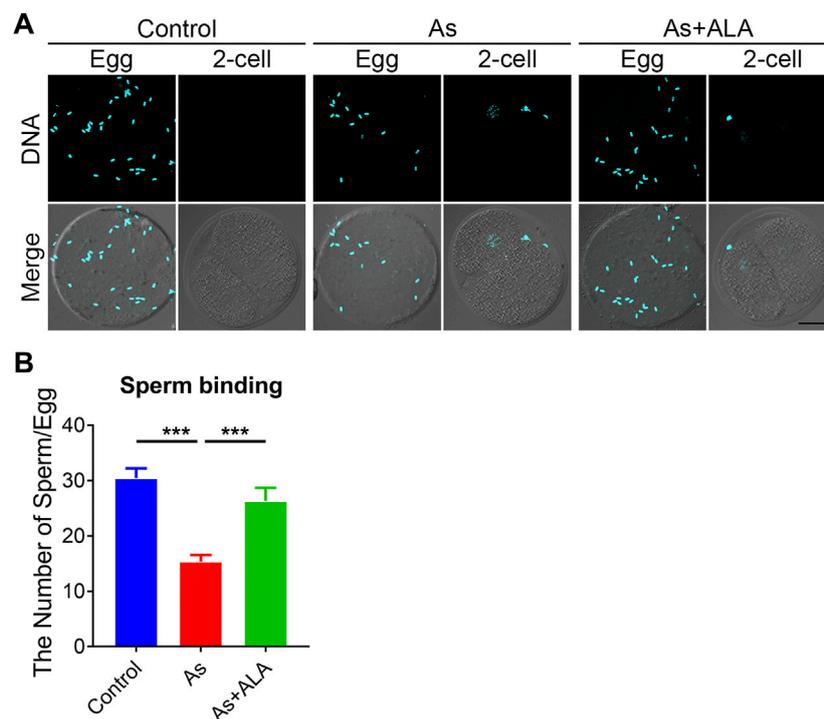
Quantitative analysis showed that actin signals were significantly decreased in arsenate-exposed oocytes in comparison to the controls, but significantly increased following ALA supplementation within expectation (control: 33.1 ± 1.7 , $n = 20$, $p < 0.001$; Arsenite: 19.5 ± 1.5 , $n = 21$; ALA: 29.5 ± 1.5 , $n = 21$, $p < 0.001$; Figures 3B,C), indicating the impaired actin dynamics induced by arsenite could be recovered by ALA.

ALA recovers the localization of cortical granules and mitochondrial integrity in arsenite-exposed oocytes

Cortical granules (CGs) exocytosis and mitochondrial dynamics are considered to be two important indicators of oocyte cytoplasmic maturation (Wang et al., 2019). Following fertilization, the cortical granules releases the contents into the extracellular space by exocytosis to prevent multiple sperm from entering the oocytes (Santella et al., 2020; Rojas et al., 2021).

Thus, we then examine whether ALA would rescue the dynamics of CGs by staining with its marker PNA-FITC. As shown in Figure 4A, CGs were localized in the subcortical region of the oocytes uniformly and continuously in the control group. In contrast, arsenite destroy the normal distribution pattern of CGs by showing the discontinued or completely disappeared signals. Quantitative analysis revealed that the fluorescence signal of CGs of the arsenite-exposed oocytes were significantly lower than that of the control oocytes, but was markedly increased following ALA supplementation (control: 26.1 ± 1.7 , $n = 28$, $p < 0.001$; Arsenite: 13.6 ± 0.4 , $n = 35$; ALA: 20.9 ± 0.6 , $n = 11$, $p < 0.001$; Figure 4B).

It requires mitochondria to provide a large amount of ATP during oocyte maturation and fertilization processes (Burke, 2017). Studies report that the impaired oogenesis and embryogenesis are highly correlated with mitochondrial dysfunction (Babayev and Seli, 2015). Thus, we further examined the effect of ALA on the mitochondrial integrity of the arsenite-exposed oocytes by staining with MitoTracker. In

**FIGURE 5**

Effect of ALA supplementation sperm binding to the zona pellucida in arsenite-exposed oocytes. **(A)** Eggs and 2-cell embryos in control, arsenite-exposed and ALA-supplemented groups were incubated (1 h) with capacitated sperm. After washing with a wide-bore pipette to remove all but two to six sperm on normal two-cell embryos (negative control), eggs and embryos were stained with DAPI. Scale bar, 30 μ m. **(B)** The number of sperms binding to the zona pellucida were calculated in control, arsenite-exposed and ALA-supplemented groups. Data were presented as mean percentage (mean \pm SEM) of three independent experiments. *** $p < 0.001$.

the control group, mitochondria were accumulated around lipid droplets in the subcortical region of porcine oocytes (Figure 4C), but lost the specific localization in arsenite-exposed oocytes. Quantitative analysis revealed that the fluorescence intensity of the mitochondrial signal was significantly reduced in the arsenite-exposed group compared to the controls (19.8 ± 1.1 , $n = 40$ vs. 28.0 ± 1.1 , $n = 26$, $p < 0.001$; Figure 4D), and rescued by the ALA supplementation as expected (23.2 ± 0.9 , $n = 26$, $p < 0.05$; Figure 4D). Taken together, the above results indicate ALA rescued the impairment of cytoplasmic maturation including the cortical granules and mitochondria of oocytes exposed to arsenite.

ALA rescues the sperm binding to the zona pellucida in arsenite-exposed oocytes

In mammals, successful fertilization depends on series of sperm-egg interaction events (Dai et al., 2017). From the beginning, the sperm binds to the extracellular matrix around the zona pellucida and only by successfully crossing the zona pellucida can it successfully fuse with the oocyte membrane to form a fertilized

egg (Oliveira-Araujo et al., 2020). To evaluate whether ALA could rescue the sperm binding ability and elevate the fertilization potential, a sperm-oocyte binding assay was performed. The sperm heads were stained with Hoechst to count the number of sperm bound to the zona pellucida. The unfertilized eggs and two-cell embryos were used as a negative control. As shown in Figure 5A, in the two-cell embryos, the zona pellucida no longer supported additional sperm binding following fertilization, because of the loss of the sperm binding site. In the arsenite-exposed oocytes, the number of sperm binding to the zona pellucida was remarkably reduced compared with the controls (15.3 ± 1.2 , $n = 29$ vs. 30.4 ± 1.8 , $n = 39$, $p < 0.001$; Figures 5A,B), but this reduction was partially restored by ALA supplementation (26.3 ± 2.4 , $n = 33$, $p < 0.001$; Figures 5A,B).

ALA reduces ROS level and DNA damage to suppress apoptosis in arsenite-exposed oocytes

Previous studies showed that arsenite has a certain oxidizing effect, leading to the increased level of ROS in

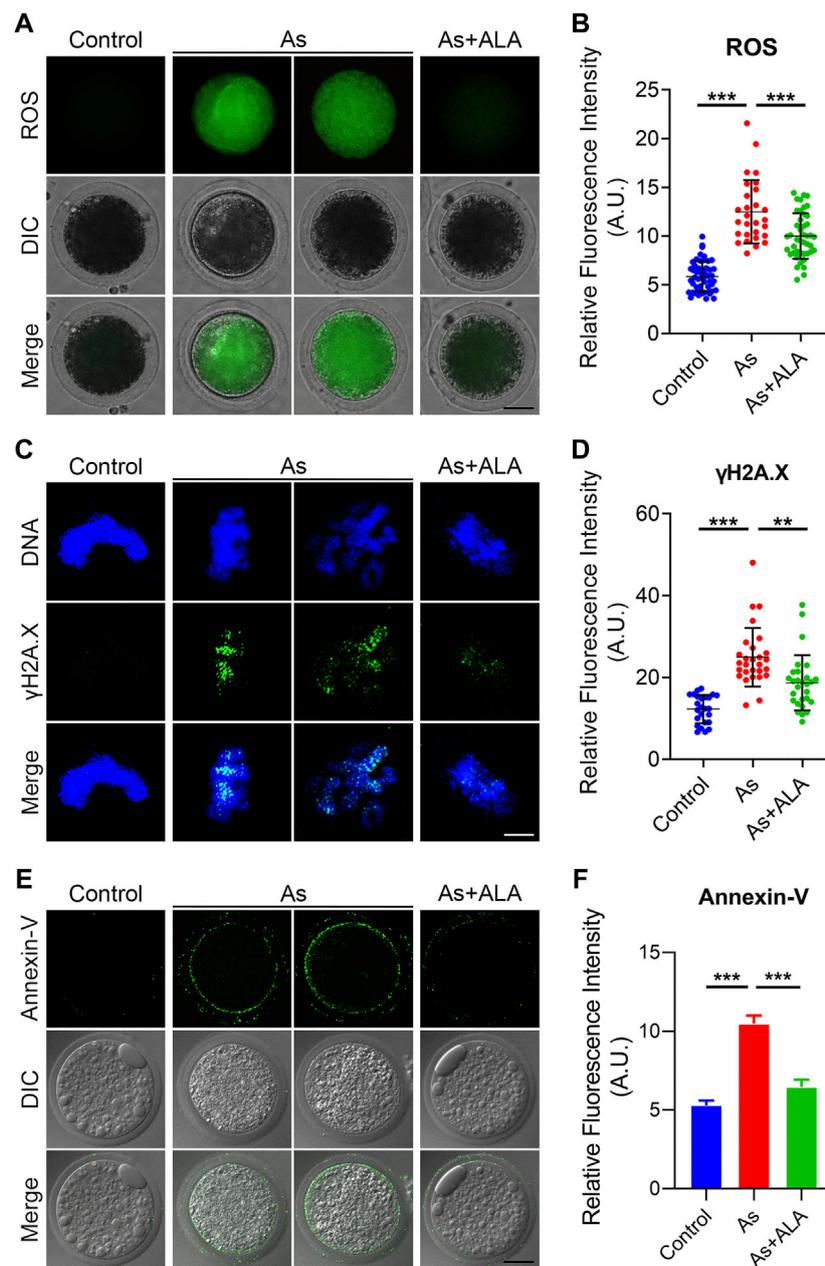
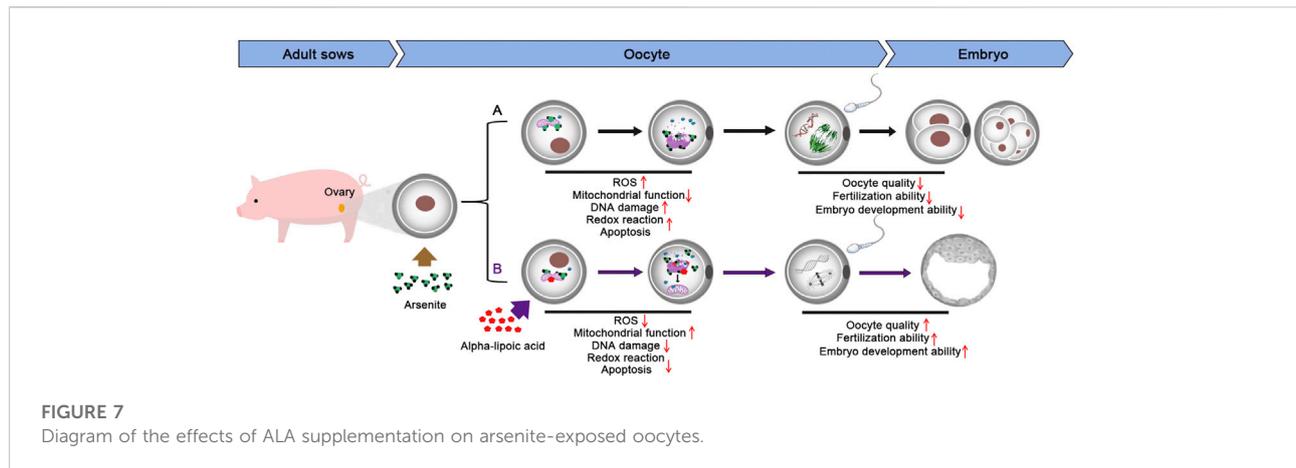


FIGURE 6

Effect of ALA supplementation on ROS levels, DNA damage and apoptosis in arsenite-exposed oocytes. **(A)** Representative images of ROS levels in control, arsenite-exposed and ALA-supplemented groups. Scale bar, 30 μm . **(B)** The fluorescence intensity of ROS were measured in control, arsenite-exposed and ALA-supplemented groups. **(C)** Representative images of DNA damage in control, arsenite-exposed and ALA-supplemented groups. Oocytes were immunostained with annexin-V-FITC. Scale bar, 2.5 μm . **(D)** The fluorescence intensity of $\gamma\text{H2A.X}$ signals were measured in control, arsenite-exposed and ALA-supplemented groups. **(E)** Representative images of apoptotic oocytes in control, arsenite-exposed and ALA-supplemented groups. Oocytes showing signals of fluorescence (green) in their membrane are regarded as early-stage apoptosis. Scale bar, 30 μm . **(F)** The fluorescence intensity of Annexin-V signals were measured in control, arsenite-exposed and ALA-supplemented groups. Data were presented as mean percentage (mean \pm SEM) of three independent experiments. * $p < 0.05$, *** $p < 0.001$.

cells of plants and animals (Srinivas et al., 2019). The accumulation of ROS further damage the DNA structures and leads to premature apoptosis and thus disrupts the normal functional development of oocytes (Zhang et al., 2020). More

importantly, ALA has been reported to be able to reduce oxidative stress to some extent in experiments on mice and human somatic cells (Hiller et al., 2016). Thus, we hypothesize that arsenite leads to the deterioration and degradation of



oocytes quality during maturation and fertilization process, and ALA could prevent the negative effect induced by arsenite in oocytes by inhibiting the ROS level, DNA damage and apoptosis. To verify this hypotheses, we measured and compared the ROS level of oocytes between the arsenite-exposed group and ALA-supplemented group with DCFH-DA Staining. The results showed that the level of ROS in arsenite-exposed oocytes was much higher than controls (12.5 ± 0.6 , $n = 28$ vs. 5.8 ± 0.2 , $n = 52$, $p < 0.001$; [Figures 6A,B](#)), and effectively decreased following ALA supplementation (10.0 ± 0.4 , $n = 44$, $p < 0.001$; [Figures 6A,B](#)).

Since the excessive oxidative stress tend to inhibit the self-repair of double-stranded DNA, leading to the massive DNA damage and consequent apoptosis ([Pisoschi and Pop, 2015](#); [Senoner and Dichtl, 2019](#)). Thus, we assessed the DNA damage by determining the fluorescence intensity by immunofluorescence staining using the anti- γ H2AX antibody. As shown in [Figure 6C](#), the fluorescence intensity of γ H2AX signals was significantly increased compared with the controls (24.9 ± 1.4 , $n = 28$ vs. 12.3 ± 0.7 , $n = 25$, $p < 0.001$; [Figures 6C,D](#)). As expected, the supplementation of ALA decreased the occurrence of DNA damage (18.7 ± 1.3 , $n = 28$, $p < 0.01$; [Figures 6C,D](#)). Meanwhile, we observed a strong fluorescent signal of Annexin-V on the plasma membrane of the arsenite-exposed oocytes, while the fluorescent signal were almost undetectable or very weak in the controls ([Figure 6E](#)). The quantitative results indicated that the fluorescence intensity of Annexin-V was significantly increased in comparison to the controls (10.5 ± 0.5 , $n = 25$ vs. 5.3 ± 0.3 , $n = 25$, $p < 0.001$; [Figures 6E,F](#)), but decreased following ALA supplementation (6.4 ± 0.5 , $n = 26$, $p < 0.001$; [Figures 6E,F](#)). Taken together, the above observations indicate that the apoptotic oocytes caused by high level of ROS and DNA damage in arsenite-exposed oocytes could be rescued following ALA supplementation.

Discussion

Female fertility is regulated by both the female reproductive system and the endocrine system ([Nicolopoulou-Stamati and Pitsos, 2001](#); [Duursen et al., 2020](#)). Arsenite, a most well-known endocrine disrupting chemicals, was reported to cause the accumulation of ROS and reactive nitrogen species (RNS) ([Davey et al., 2008](#)). Excessive ROS and RNS attack important macromolecules and organelles leading to cell apoptosis ([Kaminsky and Zhivotovsky, 2014](#); [Tapeinos et al., 2018](#)). As a strong antioxidant naturally present in the body, we hypothesize that ALA could restores the meiotic competency and fertilization capacity of porcine oocytes induced by arsenite. To verify this hypothesis and investigate the potential mechanisms, we use porcine oocytes as an experimental model. Here we aim to provide a new perspective for the application of ALA in preventing the declined oocyte quality induced by environmental EDCs.

The cumulus expansion of the COCs and the proportion of PBE are two key indicators of the porcine oocytes developmental competence ([Lan et al., 2020](#); [Du et al., 2021](#)). Our results revealed that oocytes exhibited a lower proportion of PBE with impaired expansion of cumulus cells following arsenite treatment, indicating that arsenite impairs the oocyte meiotic maturation and developmental competence. As expected, the supplementation of ALA declined the PBE rates, suggesting that ALA indeed has the potential to ameliorate the oocytes developmental competence. To further reveal whether ALA could restore the arsenite-induced decline of oocyte fertilization capacity, we then calculated the fertilization rates and blastocyst development rates of oocytes. As expected, the supplementation of ALA improved the fertilization rates and blastocyst development rates. This result suggested that

ALA indeed has the potential to ameliorate the oocyte fertilization capacity of arsenite-exposed oocytes.

To further reveal the potential mechanisms of ALA to ameliorate the declined oocytes developmental competence induced by arsenite, we next examined the critical biological events occurred during the nuclear and cytoplasmic maturation processes of oocytes. The spindle assembly and function are intimately linked to the intrinsic dynamics of microtubules, which is responsible for the accurate chromosome segregation during meiosis (Brieno-Enriquez and Cohen, 2015). Also, Spindle assembly is a crucial event for euploidy during oocyte maturation. And our current study illustrated ALA supplementation restores the defected meiotic spindle morphology and chromosome misalignment caused by arsenite. Moreover, the disruption and loss of meiotic spindle assembly is always associated with the microtubule dynamics and stability (Cleary and Hancock, 2021). Tubulin acetylation that occurs on Lys-40 of the α -tubulin subunit is found both in somatic cells and oocytes as an indicator of stabilized microtubules. Our findings further confirm these phenomena and revealed that ALA could maintain the microtubule stability and thus to ameliorate the defected spindle/chromosome structures in the Arsenite-exposed oocytes.

Actin filaments (F-actin) occur as microfilaments, which is essential component of the cytoskeleton (Duan et al., 2020). Actin cytoskeleton dynamics are critical for cellular processes during oocyte meiotic maturation, including nuclear positioning, GVBD, spindle migration, spindle rotation, chromosome segregation, and PBE (Duan and Sun, 2019). Our findings confirmed that the notably destroyed actin cytoskeleton dynamics caused by arsenite were prevented by the supplementation of ALA. The distribution and polymerization of F-actin could be another dominant mechanism by which ALA could restore the arsenite-induced oocyte meiotic failure.

Cortical granules are membrane-bound organelles located in the cortex of unfertilized oocytes, specifically exist in oocytes (Liu, 2011). Once fertilized, the cortical granules release its contents to cleave the sperm binding site ZP2, preventing polyspermy (Gahlay et al., 2010). Thus, proper dynamics of Cortical granules are often considered to be the key indicator of oocyte cytoplasmic maturation. Our data indicated that ALA supplementation recovered the abnormal distribution of CGs induced by arsenite.

In addition, mitochondrial dynamics is another important sign of oocyte cytoplasmic maturation. Mitochondria provide the ATP for cellular activities during oocytes maturation and fertilization processes (Udagawa et al., 2014). And chromosomal segregation disorders, fertilization failures and early apoptosis are always paired with mitochondrial

dysfunction (Seli et al., 2019). Our data revealed that ALA could protect the mitochondrial integrity destroyed by arsenite. These above observations confirmed that ALA could prevent the defective oocytes cytoplasmic maturation and to ameliorate the oocytes developmental competence destroyed by arsenite.

During fertilization, sperms bind to the zona pellucida and then undergo the acrosome reaction, after sperms penetrate the zona pellucida and finally fuse with the oocyte plasma membrane (Tokuhiro and Dean, 2018). So, if sperm do not bind, they cannot penetrate the zona matrix, and they cannot fuse with the plasma membrane. In addition, after fertilization, ZP2 is cleaved by ovastacin to ensure monospermic fertilization because polyspermic aneuploidy is also a significant threat to embryonic survival, and mice have developed strategies (Xiong et al., 2017). Prevention of sperm binding to the zona pellucida provides the ultimate postfertilization block to polyspermy (Vogt et al., 2019). Based on these understandings, our data found that arsenite impaired the ability of oocytes to bind sperms, and ALA supplementation is able to restore the declined numbers of sperm binding to the zona pellucida. This observation implied that ALA could restored sperm binding abilities and thus improve the fertilization potential impaired by arsenite.

Accumulating studies have indicated that oxidative stress induced by environmental endocrine disruptors might be a major cause for the deterioration of oocyte quality (Ding et al., 2020; Lombo and Herraes, 2021). Thus, we hypothesize that as a strong antioxidant, ALA supplementation could eliminate the excessive ROS, which ameliorate oocyte developmental competence and fertilization potential. Our results illustrated ALA supplementation attenuated ROS levels and thus inhibit DNA damage and apoptosis induced by arsenite.

Taken together, we provide a body of evidence that the supplementation of ALA restores, at least partially, all of the meiotic abnormalities and impaired fertilization ability induced by arsenite, through suppressing the increase of ROS level and the occurrence of DNA damage along with apoptosis caused by arsenite. Meanwhile, we demonstrate that ALA supplementation is an effective and feasible strategy to prevent the potential negative effect induced by environmental endocrine disruptors and ameliorate the oocyte quality (Figure 7).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MZ: Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing-Review; Editing and Funding acquisition; LS: Investigation, Formal analysis, Data curation and Writing-original draft; ZZ: Investigation, Data curation; LyS: Validation, Writing-review; editing; YwZ: Writing-Review; Editing; ZL: Investigation; YtZ: Investigation; YhZ: Conceptualization, Methodology, Resources, Supervision and Project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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